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Overcoming Reduced Glucocorticoid Sensitivity in Airway Disease

Molecular Mechanisms and Therapeutic Approaches

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Abstract

There is a considerable and growing unmet medical need in respiratory disease concerning effective anti-inflammatory therapies for conditions such as severe asthma, chronic obstructive pulmonary disease and cystic fibrosis. These diseases share a predominant characteristic of an enhanced and uncontrolled inflammatory response in the lungs, which contributes to disease progression, hospitalization and mortality. These diseases are poorly controlled by current anti-inflammatory therapies including glucocorticoids, which are otherwise effective in many other inflammatory conditions or in milder disease such as asthma. The exact cause of this apparent impairment of glucocorticoid function remains largely unclear; however, recent studies have now implicated a number of possible mechanisms. Central among these is an elevation of the oxidant burden in the lungs and the resulting reduction in the activity of histone deacetylase (HDAC)-2. This contributes to both the enhancement of proinflammatory mediator expression and the impaired ability of the glucocorticoid receptor (GR)-α to repress proinflammatory gene expression. The oxidant-mediated reduction in HDAC-2 activity is, in part, a result of an elevation in the phosphoinositol 3-kinase (PI3K) δ/Akt signalling pathway. Blockade of the PI3Kδ pathway restores glucocortiocoid function in both *in vitro* and *in vivo* models, and in primary cells from disease. In addition, inhibition of the PI3Kδ and PI3Kγ isoforms is anti-inflammatory in both innate and adaptive immune responses. Consequently, selective inhibition of this pathway may provide a therapeutic strategy both as a novel anti-inflammatory and in combination therapy with glucocorticoids to restore their function. However, a number of other oxidant-related and -unrelated mechanisms, including altered kinase signalling and expression of the dominant negative GRβ, may also play a role in the development of glucocorticoid insensitivity. Further elucidation of these mechanisms and pathways will enable novel therapeutic targeting for alternative anti-inflammatory drugs or combination therapies providing restoration for the anti-inflammatory action of glucocorticoids.

1. Overview of Glucocorticoid Insensitivity in Airway Diseases: Medical Need

Glucocorticoids are effective anti-inflammatory drugs that are used to control both acute and chronic inflammatory responses in a wide range of diseases. However, in some diseases the anti-inflammatory actions of glucocorticoids are impaired or ineffective. These include depression, inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, acute lymphoblastic leukaemia, cystic fibrosis (CF), idiopathic pulmonary fibrosis, severe asthma and chronic obstructive pulmonary disease (COPD).[1-7] Respiratory diseases are predominant amongst these and their incidence, particularly severe asthma and COPD, are increasing.[8,9] The problem of relative glucocorticoid insensitivity in these diseases is compounded by the fact that the precise immunological and molecular mechanisms of these diseases remain complex and unclear.[10] Current anti-inflammatory therapies for severe asthma, COPD and CF are inadequate, which highlights a substantial unmet medical need with significant disease management problems and cost burden for healthcare systems worldwide. Recent attempts to find effective anti-inflammatory alternatives, such as anti-tumour necrosis factor (TNF)-α agents for COPD, have proven unsuccessful and place some doubt on the reliability of 'single target' therapies in complex and poorly understood inflammatory diseases.[11] In addition, the incidence of a relative glucocorticoid insensitivity is seen in a wide range of diseases, yet many of these harbour 'similar' characteristics including oxidative stress and innate immunity. Therefore, the likelihood of at least some degree of mechanistic commonality is large; this remains unaddressed but would be important in developing a therapeutic strategy with a broad disease application.

In this review we aim to introduce the reader to glucocorticoids, their mechanism of action and the basic pathophysiologies of COPD, severe asthma and CF. In addition, we aim to describe the phenomenon of relative glucocorticoid insensitivity in these diseases, including proposed mechanisms and thereafter potential anti-inflammatory therapeutic strategies.

2. Glucocorticoids: Mechanism of Action

Glucocorticoids mediate a broad-ranging immunosuppression through binding and activation of the glucocorticoid receptor (GR)-α. Non-glucocorticoid bound GRα is sequestered in the cytosol by a complex of chaperone proteins including heat shock proteins 70 and 90, which prevent non-ligand-mediated GRα activity.[12,13] Association with this complex enables GRα to adopt the correct conformation to allow glucocorticoid binding.[13] Upon glucocorticoid binding, GRα dissociates from this complex and translocates to the nucleus where it mediates both repression and induction of gene expression (figure 1).[14,15] The exact contributions of GRα-mediated gene repression (transrepression) and gene activation (transactivation) to the anti-inflammatory actions of glucocorticoids remain unresolved and controversial, but may be stimulus dependent.[16] There is also increasing evidence for rapid 'non-genomic' glucocorticoid actions that may arise from (i) specific interactions with as yet unidentified cell surface receptors or (ii) direct action in the cell lipid membrane.[17,18] These non-genomic actions are proposed to lead to rapid signalling through the generation of secondary signalling messengers and thereafter influencing signalling pathways.[17] However, investigation into these non-genomic mechanisms are in their infancy and their role, if any, in either the anti-inflammatory actions of glucocorticoids or in disease is unknown. Therefore, these are not discussed in this manuscript.

2.1 Glucocorticoid-Mediated Transrepression

GRα monomers are able to associate directly with promoter-bound transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1.[19] This association allows GRα to be targeted directly to the activated promoter region of proinflammatory genes controlled by these transcription factors. Thereafter, GRα facilitates silencing of gene transcription by modulation of critical covalent modifications on the core histone proteins

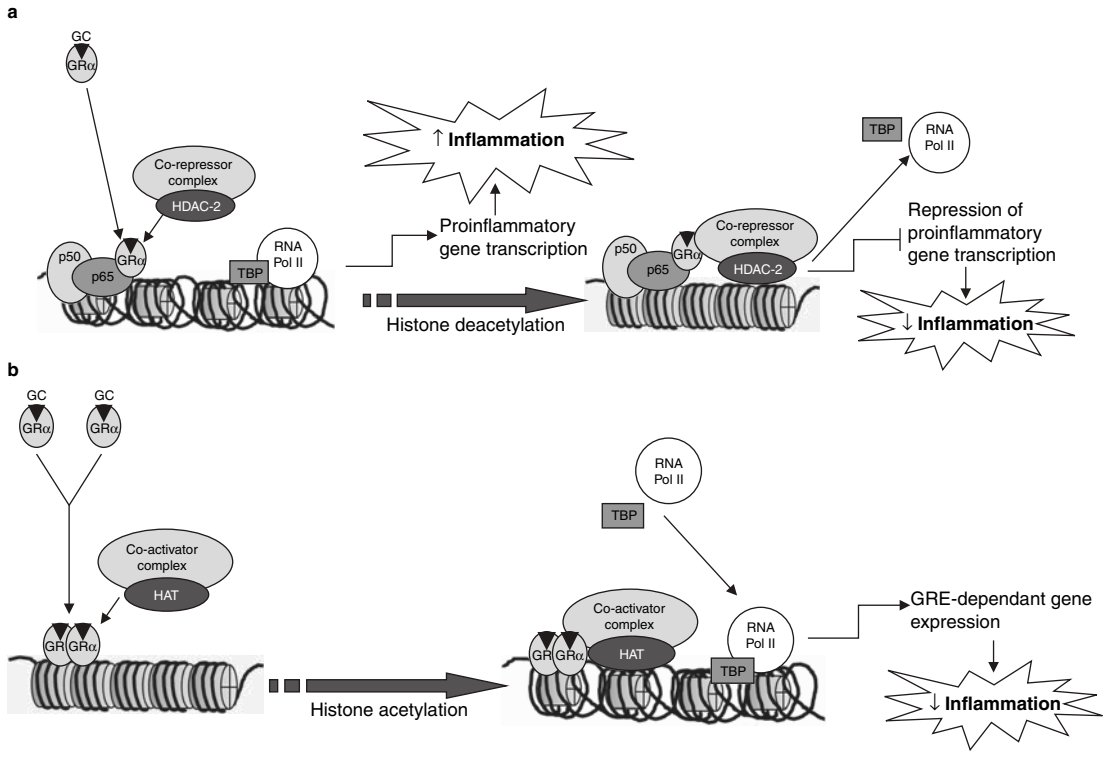

Fig. 1. Diagrammatic representation of the basic mechanism by which glucocorticoid (GC) receptor-α (GRα) [**a**] represses transcription factor-driven expression of proinflammatory genes (transrepression) and (**b**) activates gene expression from GC response element (GRE) promoter sites. Nuclear factor-κB is represented by a heterodimer of its p50/p65 subunits. **HAT** = histone acetyltransferase; **HDAC** = histone deacetylase; **RNA Pol II** = RNA polymerase II; **TBP** = TATA binding protein.

such as acetylation and methylation.[20-22] These modifications are central in mediating conformational changes that regulate access for the transcriptional machinery to the promoter region.[20] GRα itself has no intrinsic enzymatic capacity to directly modulate these histone modifications, but recruits co-repressor complexes such as mammalian Sin3a (mSin3a) and nucleosome remodelling and deacetylase/Mi2 (NuRD/Mi2) complexes.[23,24] These co-repressor complexes contain DNA and chromatin-modifying enzymes, including methyltransferases and histone deacetylases (HDACs), which 'turn off' gene transcription by altering the covalent modifications on the core histone proteins and DNA[21,25] (figure 1). The recruitment of HDAC-2 appears to be of particular importance for GRα-mediated transrepression. Specific reduction of HDAC-2 expression using small interfering RNA (siRNA) abolishes glucocorticoid-GRα-mediated repression of proinflammatory gene expression.[26] HDAC-2 induces gene silencing in a nonspecific manner by removing the acetyl groups from the amino terminal tails (deacetylation) of the core histone proteins.[20] This enables DNA to re-associate with the core histone proteins forming a compact or 'condensed' tertiary chromatin structure which dislodges the transcriptional machinery and prevents further binding.[20,27] HDAC-2 also deacetylates GRα itself, which serves to enhance its association to NF-κB.[26]

2.2 Glucocorticoid-Mediated Glucocorticoid Receptor (GR)-α Transactivation

In addition to repression of gene expression, GRα can also promote gene expression (transactivation) by forming homodimers and directly binding to the glucocorticoid response element (GRE)

promoter (figure 1). GRα-GRE binding induces the expression of a number of anti-inflammatory mediators including interleukin (IL)-10, secretory leukocyte protease inhibitor and mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1).[28,29] In addition, glucocorticoid repression of inflammatory genes under the control of NF-κB may also be partly facilitated through GRα-mediated induction of inhibitory protein κB α (IκBα) from the GRE. The resulting elevated IκB expression sequesters NF-κB to the cytosol where it is held in an inactive cytoplasmic complex.[30]

Particular attention has been paid to the glucocorticoid-dependent induction of MKP-1. Induction of the potent proinflammatory p38 MAPK/NF-κB signalling pathway can regulate GRα function (via p38 MAPK phosphorylation; discussed in sections 2.4 and 4.3), which is in turn regulated by GRα through induction of MKP-1 expression.[31,32] Adenoviral overexpression of MKP-1 reduced p38 MAPK and NF-κB activation, and the expression of CXC chemokine ligand 8 (CXCL8).[31] In addition, siRNA-mediated knockdown of MKP-1 partially reverses dexamethasone repression of TNFα-mediated activation of p38 MAPK. TNFα-induced NF-κB-mediated proinflammatory gene expression is repressed by dexamethasone in the presence of MAPK kinase 6 (MKK6), a p38 MAPK inhibitor; however, blocking MKP-1 by addition of MKP-1 siRNA diminishes this effect.[31] These studies provide evidence for the direct importance of GRα transactivation in mediating the anti-inflammatory actions of glucocorticoids.

However, GRα-mediated gene expression is also thought to be responsible for many of the unacceptable side effects seen with high doses of glucocorticoids and is a common reason for the withdrawal of high-dose oral glucocorticoids as an anti-inflammatory therapy.

2.3 Relative Contribution of GRα Transrepression and Transactivation in Glucocorticoid Inflammatory Mediator Suppression

GRα transactivation is widely associated with the unacceptable side effects seen at higher doses of glucocorticoids. Therefore, the relative contributions of both transrepression and transactivation may be therapeutically important if these mechanisms can be selectively targeted. Transgenic mice expressing a mutant GRα, which is unable to dimerise and thereby bind to the DNA ($GR\alpha^{dim}$ mice) are able to repress inflammatory responses induced by both local and systemic lipopolysaccharide exposure comparable to wild-type animals.[16] Therefore, it was proposed that GRα DNA binding and consequent induction of gene expression (transactivation) was non-essential for the anti-inflammatory actions of GRα. However, further investigations using these mice in a model of contact allergy demonstrated that the anti-inflammatory response of glucocorticoids was impaired in the $GR\alpha^{dim}$ mice.[33] This suggests that the relative contributions of GRα transrepression and transactivation may depend on the inflammatory stimulus.

To date, more importance has been placed on GRα transrepression in glucocorticoid-mediated inflammatory repression. This is principally due to greater experimental investigation into GRα-mediated transrepression and concern over the transactivational-related adverse effects rather than substantial experimental evidence showing a secondary/minor role for GR-mediated transactivation in the glucocorticoid-mediated anti-inflammatory action. Therefore, further, more direct investigation is needed to decipher the real contribution of GRα-meditated transactivation in a range of inflammatory responses. Critically, this must also be done using human cells and tissues as murine cells do not express the GRβ isoform, which may play an important role (discussed in sections 2.5 and 4.4). Investigation in primary human cells and tissues using well characterized dissociated corticosteroids may resolve this issue in the near future.

2.4 Regulation of GRα Function by Phosphorylation

Phosphorylation plays an important role in the regulation of GRα function.[34,35] Human GRα is phosphorylated on at least five serine residues (S113, S141, S203, S211 and S226), which

are positioned within the major transactivational domain (AF1).[34] Although the exact functional effects of phosphorylation at these sites are unclear, a number of studies have shown how they may determine the activity of the AF1 domain on GRα. Phosphorylation of S211 and S226 is thought to be important for the transcriptional activity of GRα, whilst S203 may be involved in GRα translocation.[35,36] Phosphorylation of S211 and S226 may also enhance the interaction of GRα with co-regulators, including the vitamin D_3 receptor-interacting protein/thyroid receptor-associated protein (DRIP/TRAP) complex and the vitamin D receptor interacting protein 150 (MED14), as well as potentially influencing GRα activity in a gene-selective manner.[35]

An array of kinases facilitates phosphorylation of GRα at distinct sites, thereby integrating GRα activation in cell-signalling pathways. These include extracellular-regulated kinase (ERK)-2, p38 MAPK, cyclin-dependent kinases (CDKs) 1, 2 and 5, glycogen synthase kinase 3 (GSK3) and c-Jun N-terminal kinase (JNK).[36-38] These kinases mediate phosphorylation that may be either activational (CDK1, 2 and 5) or inhibitory (JNK, GSK3). In addition, certain kinases such as p38 MAPK appear to facilitate both activation and inhibition of GRα activity in a cell-specific manner.[36,39]

2.5 A Role for GRβ?

The human glucocorticoid gene encodes two isoforms of the GR receptor generated by splice variants resulting in expression of the GRα and GRβ isoforms.[40] In contrast to the canonical GRα, GRβ is an orphan receptor which is localized to the nuclear compartment and is devoid of the ability to bind glucocorticoids.[41] The sole function of GRβ was thought to be as a dominant negative regulator of GRα through competition for co-regulators including the GR interacting protein 1 (GRIP-1).[40,42] However, although the GRβ isoform lacks the ability to bind to glucocorticoids, recent evidence suggests that, in addition to its dominant negative regulatory role for GRα function, GRβ may also facilitate direct transcriptional effects of its own. Although no endogenous ligands for GRβ have been identified, Cidlowski and colleagues[41] demonstrated that GRβ is also localized in the cytosol using COS-1 and U-2 OS cells and has the ability to bind the synthetic GRα receptor antagonist mifepristone (RU486), which mediated nuclear translocation of GRβ. Interestingly, in the absence of GRα, microarray analysis showed that GRβ was capable of regulating gene transcription, which was diminished upon binding of mifepristone.[41] Additional studies using Hela cells have also shown that GRβ has intrinsic gene-specific transcriptional activity (both negative and positive), where the majority of affected genes were distinct from those regulated by GRα.[43] These recent findings clearly demonstrate that the role of GRβ may be more complex and important than its perceived single role as a dominant negative regulator of GRα. The further complication of additional splice variants with potentially distinct functions also needs further examination.[44] However, these are novel discoveries and further investigations, particularly in primary human cells, are needed to elucidate the extent and role of these proposed additional GRβ actions as well as any cellular or tissue specificity.

3. Relative Glucocorticoid Insensitivity in Respiratory Disease

3.1 Severe Asthma

Allergic responses in the airways of patients with asthma may induce narrowing of the large airways that is largely reversible by bronchodilator drugs such as short- and long-acting β_2 agonists. The allergic response in asthma is characterized by an adaptive/T helper (T_h) type 2 cell-mediated response that induces an isotype switch in B cells for the production of immunoglobulin (Ig) E. This in turn initiates mast cell degranulation through activation of high-affinity IgE receptors expressed on the mast cells, and also through the induction of eosinophilic activation and responses.[10] Low doses of inhaled glucocorticoids effectively control the inflammatory response in most patients with asthma

and are considered a first-line treatment.[45] However, a small proportion of asthmatic patients have a severe form of asthma that is not adequately controlled by existing asthma medications, including glucocorticoids, even when used at high doses.

The inflammatory response in the lungs of patients with severe (relatively glucocorticoid-'insensitive' or -'unresponsive') asthma is not different to that observed in the lungs of patients with mild/moderate (relatively glucocorticoid-'sensitive' or -'responsive') asthma, as demonstrated by an elevation in both eosinophil and leukocyte numbers in bronchial biopsies from patients with severe asthma.[46] It is clear that patients with severe asthma are treated with much higher doses of glucocorticoids, which therefore gives rise to the idea of relative glucocorticoid insensitivity.[47] This relative glucocorticoid insensitivity is supported by observations that glucocorticoids fail to repress the release of pro-inflammatory mediators from both circulating inflammatory cells (peripheral blood mononuclear cells [PBMCs]) and lung macrophages (obtained from bronchoalveolar lavage) induced by lipopolysaccharide from patients with severe asthma as compared with patients with mild/moderate asthma, including CXCL8, TNFα and granulocyte macrophage-colony stimulating factor (GM-CSF).[48,49] The ratio of matrix metalloprotease (MMP)-9 to the tissue inhibitor of MMP (TIMP)-1 is higher in the lungs of patients with severe asthma.[50] Glucocorticoids elevate TIMP1 expression but this response is absent in alveolar macrophages from patients with severe asthma, which may contribute to the abnormal tissue remodelling in the airways of severe asthma leading to a reduction in lung function and β-agonist reversibility in these patients.[2,50] Indeed, Macedo et al.[46] have recently shown that there is a greater degree of subepithelial fibrosis and airway smooth muscle in patients with severe asthma as compared with non-severe asthma.

Interestingly, the inflammatory component of severe asthma may have some similarities to the inflammatory response seen in COPD.[10] This is particularly apparent with the presence of an innate/T_h1 response in addition to the T_h2 cells that predominate in mild/moderate asthma as well as more CD8+ T cells.[10,51-53] In contrast to mild/moderate asthma, patients with severe asthma also have increased neutrophils in their sputum, increased CXCL8 and TNFα, and an elevated oxidant burden in the lung.[51,54-57]

Asthmatic patients who smoke (accounting for ~25% of asthmatic patients) also have a reduced response to inhaled and oral glucocorticoids, and develop more severe asthma with an associated decline in lung function compared with asthmatic patients who do not smoke.[58,59] These smoking asthmatic patients also have an increase in sputum neutrophils and CXCL8, which is more closely associated with severe asthma and COPD than with mild/moderate asthmatic patients who do not smoke.[59] The evidence for this smoking-related effect is further found in COPD (discussed in section 3.2) where the major aetiological factor is cigarette smoke, which may also contribute to the development of the relative glucocorticoid-insensitivity seen this disease.[2,60]

3.2 Chronic Obstructive Pulmonary Disease (COPD)

COPD is characterized by airflow limitation that is progressive, poorly reversible and is associated with airway remodelling and an enhanced chronic inflammation in the lungs.[61] Although cigarette smoke is the main aetiological factor in the development of COPD, the exact mechanism and contributing factors involved in the development of COPD are complex and not well understood. Airflow limitation is a central feature of both COPD and asthma; however, an important defining factor differentiating these two diseases is that the airflow limitation in asthma is largely reversible, whereas it is largely irreversible in COPD.[62] A seminal study from Hogg and colleagues[62] identified a potential cause of the airflow obstruction in COPD to be remodelling of the small airways, which correlated with disease progression.

Another defining factor between COPD and asthma is the type of underlying inflammation in the lungs. The inflammation in the lungs of COPD patients is predominantly driven by an

innate/T_h1 type immune response, whereas in asthma it is a predominantly adaptive/T_h2 immune-mediated response.[10,60] The chronic enhanced lung inflammation in COPD is associated with an elevation in the number of macrophages, neutrophils, T_h1 and type 1 cytotoxic T (Tc1) cells and CD8+ T cells in the lung.[10,51,60] In addition, COPD patients have mucus hypersecretion and reduced mucociliary clearance, which is a major contributing factor in bacterial colonization and infection resulting in exacerbations as well as varying degrees of emphysema (alveolar wall destruction) and chronic bronchitis.[60,63,64]

Unlike asthma patients, the majority of patients with COPD respond very poorly to inhaled and oral glucocorticoids, even at high doses. Glucocorticoid treatment in patients with COPD has no impact on the rate of decline in lung function, disease progression or mortality. There is evidence that glucocorticoids may have a modest beneficial effect on the number of exacerbations, although this has recently been questioned.[65,66] The inflammatory response seen in the lungs of COPD patients is glucocorticoid insensitive. A number of studies have shown that inhaled glucocorticoids have no impact on the numbers of inflammatory cells in the lungs or on the release of proinflammatory mediators in COPD patients.[29,67,68] In addition, glucocorticoids fail to repress the release of proinflammatory mediators from alveolar macrophages obtained from the bronchoalveolar lavage from COPD patients.[69]

3.3 Cystic Fibrosis

CF is one of the most common lethal genetic diseases in Caucasians, and is characterized by an aggressive and predominantly innate/T_h1 inflammatory response with extensive destruction of the lungs.[70] In contrast to both COPD and severe asthma, the underlying cause of CF is known. The development of CF is a result of a mutation in the gene that encodes the CF transmembrane conductance regulator (CFTR).[70] The CFTR is expressed in epithelial cells and leukocytes, and acts mainly as a chloride channel, although it has a plethora of other key regulatory functions, including regulation of adenosine triphosphate channels, intracellular vesicle transport, acidification of intracellular organelles, and inhibition of both epithelial sodium channels and endogenous calcium-activated chloride channels.[70] The development of CF is often rapid; the lungs of children with CF appear normal at birth, but then rapidly become infected and inflamed. The influx of inflammatory polymorphonuclear cells is associated with an imbalance in pro- and anti-inflammatory mediators including an elevation in NF-κB signalling, and increased IL-6, CXCL8, TNFα and ecosanoid expression, whilst the levels of anti-inflammatory mediators such as IL-10, lipoxin and docosahexaenoic acid are reduced.[70-73] Despite the importance of the inflammatory response in the pathogenesis of CF, the current therapies are ineffective. Similarly to COPD and severe asthma, glucocorticoids are ineffective at controlling the aggressive inflammatory response seen in CF. Ibuprofen is commonly used as an alternative anti-inflammatory therapy as it is inexpensive, has relatively few adverse effects, and has been shown to be beneficial when given before the onset of severe inflammation and pathological changes in the lung.[74] However, due to the aggressive nature of the inflammatory response in CF and its undoubted importance in the progression of the disease, the lack of an effective broad-ranging and powerful anti-inflammatory drug such as a functional glucocorticoid-type drug represents a major unmet medical need.

4. Underlying Mechanisms of Glucocorticoid Insensitivity

The molecular mechanisms of the relative glucocorticoid insensitivity in these respiratory diseases remain both unclear and understudied. Although there is a recognised inherent variation in glucocorticoid responsiveness between different cell types and tissues, the majority of cells that make up the lung and the infiltrating inflammatory cells are responsive to glucocorticoids.[75] One exception to this are neutrophils, which are less responsive to glucocorticoids (discussed in detail in section 4.5). Furthermore, many other inflammatory conditions involving many of these

cell types are well controlled. It is therefore unlikely that an intrinsic relative glucocorticoid unresponsiveness of a particular cell type or of the lung itself can fully account for the reduction of glucocorticoid-insensitivity seen in CF, severe asthma and COPD.

There may also be some commonality in the mechanisms of glucocorticoid insensitivity between these diseases and indeed in other diseases that respond relatively poorly to glucocorticoids. Common immunological features of severe asthma, COPD and CF include an enhanced inflammatory response that either has components of or is predominantly driven by the innate/T_h1 type immune response. The innate/T_h1 response itself is well documented as responding well to glucocorticoids; however, an 'enhanced' inflammatory response may incorporate an overriding factor. One of the more prominent theories implicated is elevated or 'overriding' oxidant burden in the lungs[60,76] (figure 2). This concept is attractive not only from the experimental and mechanistic evidence, but also by its alignment with other proposed mechanisms. However, severe asthma, COPD and CF are often studied separately, whereas an approach to dissect the pathways governing glucocorticoid function across these diseases may prove more fruitful. Other proposed mechanisms, both distinct and aligned with oxidative stress, include genetic factors,

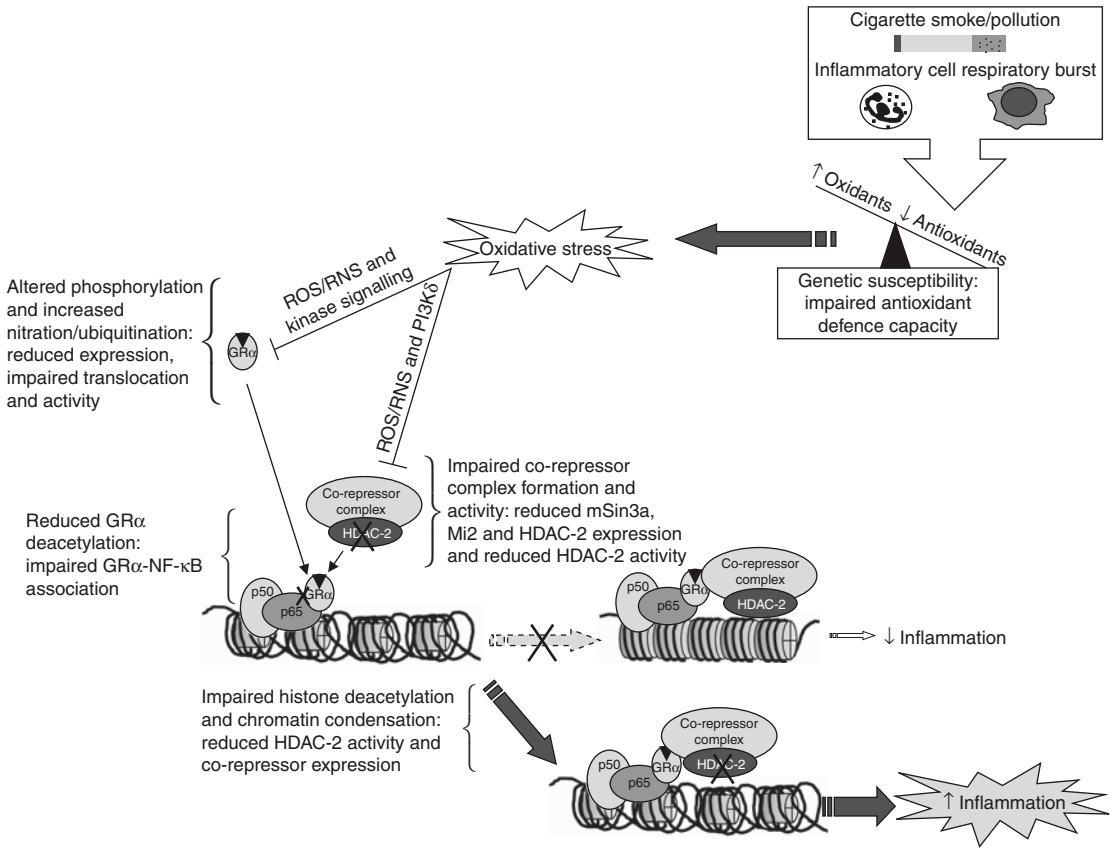

Fig. 2. Diagrammatic representation of the proposed mechanisms by which oxidative stress impairs glucocorticoid receptor-α (GRα) function leading to an enhanced proinflammatory response that is resistant to glucocorticoid-mediated repression. Nuclear factor (NF)-κB is represented by a heterodimer of its p50/p65 subunits. **HDAC** = histone deacetylase; **mSin3a** = mammalian Sin3a; **RNS** = reactive nitrogen species; **ROS** = reactive oxygen species.

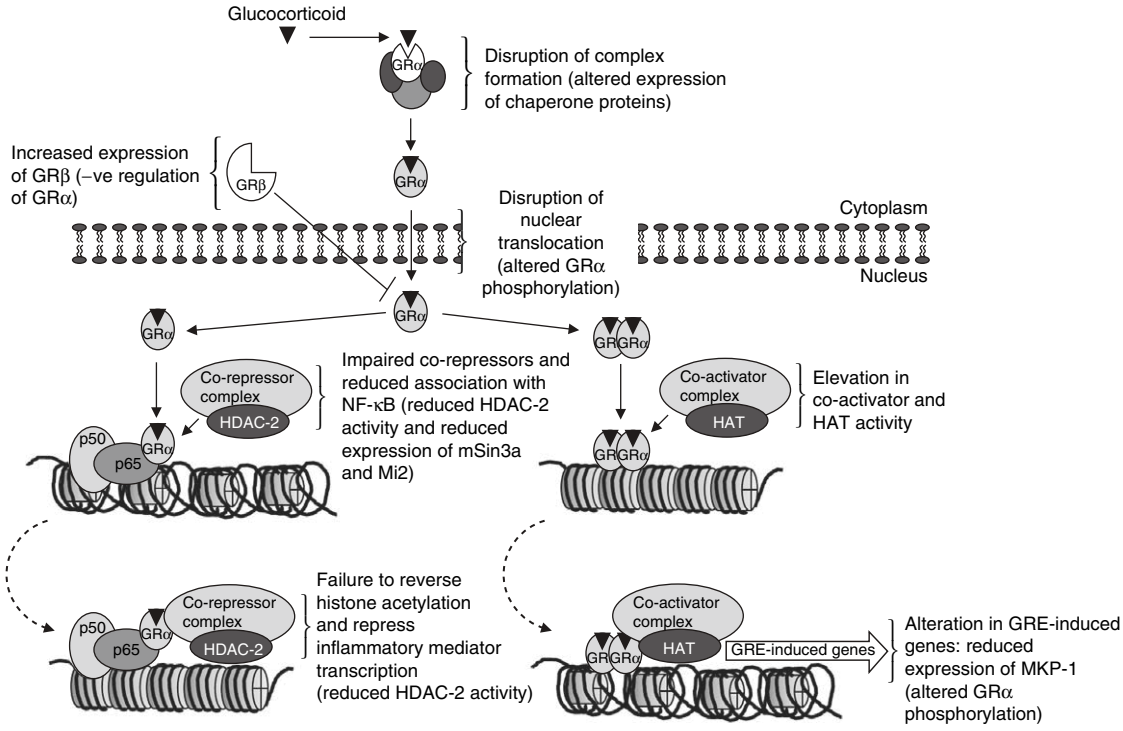

Fig. 3. Diagrammatic representation of the proposed mechanisms by which glucocorticoid receptor-α (GRα) function may become defective in airways disease leading to glucocorticoid insensitivity. Nuclear factor (NF)-κB is represented by a heterodimer of its p50/p65 subunits. **GRE** = glucocorticoid response element; **HAT** = histone acetyltransferase; **HDAC** = histone deacetylase; **MKP-1** = mitogen-activated protein kinase phosphatase 1; **mSin3a** = mammalian Sin3a.

altered kinase signalling, elevated GRβ expression and alteration of the histone acetylation/deacetylation balance (figure 3).

4.1 Oxidative Stress and Histone Deacetylase Activity

An elevated oxidant burden in the lungs is a major component of severe asthma, COPD and CF, which may be a central factor in the development of relative glucocorticoid insensitivity in these diseases.[60,76] This oxidant stress may be derived from both exogenous sources such as pollution and cigarette smoke and endogenously from the respiratory burst of proinflammatory cells such as macrophages and neutrophils. Under physiological conditions, oxidants and the redox state of the cell are an integral part of cellular signalling and function, including that of GR.[77,78] However, a significant elevation in oxidants can overcome both intra- and extracellular antioxidant defences, and can alter signalling pathways and protein function.[79] The role of additional exogenous oxidative stress such as cigarette smoke is highlighted by both its induction of reduced glucocorticoid insensitivity in experimental models, the development of glucocorticoid insensitivity in COPD and the development of relative glucocorticoid insensitivity in patients with mild/moderate asthma who smoke.[58,60,80,81] Oxidative stress also plays a key role in the inflammatory processes associated with CF.[82]

A central factor in both an oxidant-mediated enhancement of inflammatory responses and development of relative glucocorticoid insensitivity may be facilitated by a direct alteration of the acetylation-deacetylation balance of the core histones.[83] Oxidative stress leads to a reduction of HDAC-2 activity (acute exposure) with an

additional reduction in HDAC-2 expression (seen with more chronic exposures) in *in vitro* and *in vivo* models.[80,84,85] The reduction in both activity and expression results from covalent modifications, including hyperphosphorylation, nitration and carbonylation mediated by reactive oxygen species (e.g. reactive carbonyls, reactive nitrogen species), and by kinase signalling pathways activated by the oxidative stress.[83,86] These modifications directly result in the reduction HDAC-2 activity and also reduce its expression by marking it out for proteasomal degradation.[84,85,87-89] As histone acetylation is a key factor in gene transcription, this oxidant-mediated loss of HDAC activity and/or elevated histone acetyltransferase (HAT) activity is likely to contribute to the enhanced inflammatory responses in these diseases (figure 2).

HDAC-2 activity and expression is reduced in both COPD and severe asthma, with the reduction of activity in the peripheral lungs of COPD patients correlating with disease severity.[49,90,91] Importantly, HDAC-2 is fundamental for functional GRα transrepression of proinflammatory genes, and a reduction in its activity/expression mediated either by oxidative stress or by siRNA severely impairs GRα transrepression of proinflammatory mediators.[26] Conversely, protection or restoration of HDAC-2 activity in *in vitro* and *in vivo* models restores glucocorticoid responsiveness.[81,92] Therefore, the reduction in HDAC-2 activity and expression seen in COPD and severe asthma is likely to be an important factor in the mechanisms of the relative glucocorticoid insensitivity seen in these diseases (figure 2).

Total HDAC activity measurement in CF has only been performed as part of one study and there was no alteration compared with healthy lungs.[91] However, this study was performed on total lung extracts and alterations may be more local or cell-type restricted, which may be missed in a relatively crude total lung analysis. Indeed, recent studies have shown that the loss of CFTR may result in a reduction of HDAC-2 activity in airway epithelial cells.[93] In addition, oxidative stress-induced hyperacetylation of the CXCL8 promoter in CF airway cell models and HAT activity may be elevated in the peripheral lungs of CF patients.[91,94] However, with so few studies in CF relating to the HDAC/HAT balance, the evidence of a role for HDAC-2 activity in the relative glucocorticoid insensitivity in CF appears promising but is not yet conclusive. This highlights a critical need for further investigations relating to glucocorticoid insensitivity in this disease.

4.2 Genetics

A number of studies have linked genetic mutations and alterations in cellular expression with the development of glucocorticoid insensitivity, as elegantly reviewed by Gross et al.[95] Early studies identified a higher incidence of relative glucocorticoid-insensitive asthma within families, which indicated that there may be a genetic link to the development of glucocorticoid insensitivity.[96] Genetic analysis of PBMCs have identified 11 genes that discriminate between relative glucocorticoid-insensitive and glucocorticoid-sensitive asthmatic patients, including those for several asthma-related proinflammatory signalling pathways, suggesting that there may be a genetic factor in the development of relative glucocorticoid-insensitive asthma.[97] A link between genetic factors and the development of relative glucocorticoid insensitivity in COPD has not been directly assessed; however, there is evidence that genetic susceptibility is likely to play a role. Studies investigating the antioxidant capacity in the lungs of COPD patients and smokers suggest that subjects who develop COPD have a reduced capacity to elevate their antioxidant defences, which may, in part, account for the development of COPD in a subset of smokers (~20%).[98-100] Furthermore, given the likely central role for oxidant stress in the development of glucocorticoid insensitivity, the reduced antioxidant capacity is also likely to contribute to the development of a relative glucocorticoid insensitivity in COPD.

The genetics of severe asthma versus mild/moderate asthma are currently being intensively investigated, but as of yet there has been no indication of genetic link with antioxidant capacity that may thereafter play a role in glucocorticoid insensitivity. In CF, the onset and level of the inflammatory response and ensuing lung

destruction is aggressive and a clear driving factor is represented by the mutation of the CFTR gene. Therefore, unlike the slow progression and development of COPD where, in addition to other factors, the oxidant imbalance may take many years to be overcome, an underlying genetic susceptibility in CF relating the antioxidant capacity is unlikely to play a central role in either the development of the disease or glucocorticoid insensitivity.

4.3 Kinase Signalling

The activation of signalling kinases is a fundamental part of physiologically controlled inflammation. However, many of these kinases such as p38 MAPK, GSK-3β, ERK1/2 and JNK also regulate GRα activity and may also influence its gene specificity.[101,102] The activity of ERK1/2, JNK and p38 MAPK are elevated in severe asthma compared with mild/moderate asthma and the elevation of p38 MAPK has also been linked to elevated phosphorylation of GRα leading to reduced ligand-binding affinity.[103,104] COPD patients also display altered kinase signalling with an elevation in the activity of both p38 MAPK and JNK compared with healthy smokers.[105,106] Elevated p38 MAPK may also be directly responsible for reduced GRα function in severe asthma through a reduction in the expression of MKP-1.[49] Altered kinase signalling may also diminish GRα function indirectly through impairment of HDAC-2 activity by elevated PI3Kδ signalling in COPD.[81,107]

In CF, the absence of the CFTR induces an intrinsic activation of proinflammatory transcription factors including AP-1 and NF-κB by mechanisms involving ERK1/2 and IKK signalling pathways.[108] This activation is then consequently sustained via autocrine stimulation by proinflammatory mediators such as IL-1β.[108] Both ERK1/2 and p38 MAPK may also be involved in the enhanced proinflammatory response in CFTR defective cells.[109] Further studies are needed to directly investigate the activation of these kinases in CF in relation to altered GRα function and glucocorticoid insensitivity, and the negative feedback regulatory mechanisms utilised by GRα such as MKP-1 expression.

4.4 GRβ Expression

Classically, GRβ is perceived as a negative regulator of GRα and its level of expression would thereby dictate the relative functional effectiveness of GRα. Consequently, the expression of GRβ correlates with effective glucocorticoid function, particularly in cells/tissues.[75,110] It is therefore likely that a high GRβ to GRα ratio in disease would impair GRα function contributing to glucocorticoid insensitivity. However, the level of GRβ in disease is a controversial area. Glucocorticoid insensitivity in severe asthma was initially found to be independent of GRα and GRβ expression.[111] Other studies have shown a relationship between GRβ expression and severe asthma.[112] In COPD, the expression of GRα is reduced with no elevation of GRβ levels.[81,113] No studies have looked at the expression of GRα or GRβ in CF. Therefore, with so few studies assessing the relative expressions of GRα and GRβ in these diseases it is difficult to assign a role for GRβ in the development of glucocorticoid insensitivity with any confidence. More studies are therefore needed to clarify their relative expression in disease.

A role for GRβ may also be found in light of recent evidence that it has an intrinsic transcriptional activity and may also be able to bind ligands.[41,43] In light of these novel and important observations, the role of GRβ in primary human cells and tissues, including those from disease, must be established. Thereafter, any potential impact on glucocorticoid insensitivity may be assessed with more confidence than a simplistic dominant negative regulation of GRα.

4.5 Neutrophils and Neutrophilic Inflammation

The majority of cells involved in the pathogenesis of lung diseases are responsive to glucocorticoids. However, neutrophils and neutrophilic inflammation are relatively unresponsive to glucocorticoid-mediated immunosuppression compared with other cell types.[81,114,115] Studies have shown a relatively higher expression of GRβ and a further induction of GRβ expression upon stimulation, which could thereafter impair the functional

effectiveness of GRα and contribute to a relative reduction in glucocorticoid sensitivity.[110] However, other studies have shown low expression of GRβ and therefore the precise relative expression levels of both GRα and GRβ in neutrophils remains both unresolved and controversial.[116]

Importantly, neutrophil-associated exacerbations of severe asthma and COPD are also poorly controlled by glucocorticoids.[114,115] However, it has not been determined whether this is an extension of the existing glucocorticoid insensitivity or if there is an additional unresponsiveness mediated by the acute elevation in neutrophils during the exacerbation. It is therefore feasible that the presence of neutrophils in both the stable and acute inflammatory responses in severe asthma, COPD and CF may contribute, in part, to their overall reduced responsiveness to glucocorticoid treatment.

Despite an apparent lack of glucocorticoid-mediated immunosuppression in neutrophils and neutrophilic inflammation, glucocorticoid treatment of neutrophils elicits GRα-dependent responses, most notably a delay in their constitutive apoptosis.[117,118] These studies demonstrate that glucocorticoids are able to mediate important GRα-specific responses in neutrophils. Induction of these responses may have important clinical implications, particularly in severe asthma and COPD, which despite being relatively glucocorticoid insensitive, is still treated with high levels of glucocorticoids. Further investigations are needed to depict the relevance and possible roles of these GRα-dependent responses when treating a neutrophil-associated inflammatory response with glucocorticoids.

4.6 Impairment of GRα Transrepression or Transactivation, or Both?

Most functional studies concerning glucocorticoid insensitivity in models mimicking aspects of airway diseases such as asthma and COPD look at the ability of glucocorticoids to repress the pro-inflammatory genes, co-factors concerned with GRα transrepression or the inflammatory cell influx.[26,49,80,81,90,92] A failure of glucocorticoids to repress these inflammatory factors is seen as glucocorticoid insensitivity. However, alterations in GRα transactivation in patients with relative glucocorticoid insensitivity has not been extensively assessed and may also be important, particularly as the potential contribution of GRα transactivation to the full anti-inflammatory actions of glucocorticoid in responsive airway diseases has not been established. Of course, the major reason that patients have limited or are withdrawn from glucocorticoid treatment is unacceptable side effects, which in turn are classically attributed to GR transactivation. Therefore, this would suggest that GRα transactivation is functional in these relative glucocorticoid-insensitive patients. However, recent evidence suggests that the regulation of specific genes by GRα may be dependent on GRα phosphorylation at specific sites.[40,101,102] It could therefore be speculated that the phosphorylation status of GRα is altered in relative glucocorticoid-insensitive patients in such a manner that transcription of genes associated with its anti-inflammatory action are impaired but not those largely associated with mediating glucocorticoid-mediated adverse effects. Furthermore, studies using the $GR\alpha^{drim}$ mice, which are essentially devoid of GRα transactivation, show that these mice still have adverse effects from glucocorticoid treatment.[119] Therefore, we cannot rule out both a substantial contribution of GRα transactivation to glucocorticoid-mediated immunosuppression in glucocorticoid-responsive airway diseases and thereafter any impairment of GRα transactivational immunosuppression in glucocorticoid-insensitive airway diseases. It is therefore essential that further studies attempt to depict the role of GRα transactivation in airway diseases as this may then influence future therapeutic strategies. Investigation using recently described dissociative glucocorticoids in patients with severe asthma and COPD may resolve many of these issues.[120]

5. Therapeutic Approaches to Overcome Glucocorticoid Insensitivity in COPD and Asthma: Alternative Treatments and Restorative Approaches

5.1 Alternative Anti-Inflammatory Therapeutic Targets

Selective inhibition of single inflammatory mediators that are thought to be important in

COPD and asthma have not proven successful.[11] This is likely to be due to the complex nature of the inflammatory responses in these diseases where removal of proinflammatory signalling derived from a single proinflammatory mediator may simply be compensated by the multitude of downstream intracellular signalling networks activated by the overall inflammatory response. Therefore, an effective alternative anti-inflammatory strategy must be directed against a target that has broad-ranging anti-inflammatory effects such as those seen with glucocorticoids. One attractive strategy that is being investigated is the inhibition of selective protein kinases, which act as signalling messengers for multiple inflammatory stimuli. These kinases are a pivotal part of multiple interconnecting inflammatory pathways and, therefore, regulate the expression of a plethora of different proinflammatory mediators.[121,122] An obvious hurdle with targeting these kinases is that many are ubiquitously expressed and in addition to inflammatory signalling are also involved in critical physiological cellular processes. However, specific inhibition of selected isoforms and the relatively unique opportunity to deliver therapies locally to the lung may provide an avenue to overcome possible toxic effects. Two such proteins kinase targets, phosphoinositol 3-kinase (PI3K) and p38 MAPK kinase, are discussed in the following sections.

5.1.1 p38 Mitogen-Activated Protein Kinase

There are four p38 MAPK isoforms, α, β, γ and δ, which are encoded by separate genes and are expressed in a tissue-dependent manner. p38 MAPK is a central mediator in many of the inflammatory signalling pathways, including activation of NF-κB, regulation of GRα and marking out proinflammatory genes for early gene transcription through phosphorylation of histone 3 at serine 10.[123] The specific roles of the individual p38 MAPK isoforms in the inflammatory response are unknown due to a lack of selective pharmacological tools; however, selective inhibition of the p38 MAPK α isoform potently inhibits the release of many proinflammatory cytokines including TNFα, IL-6, CXCL-8 and GM-CSF.[124,125] Many of these pathways and genes are prominent in the relatively glucocorticoid-insensitive inflammatory responses seen in severe asthma, COPD and CF.[51] Selective inhibition of p38 MAPK also reduced the inflammatory response in nasal biopsies from CF patients, including CXCL8 release, COX-2 upregulation and neutrophil migration, although the contribution of the individual p38 MAPK isoforms has yet to be assessed.[126] Therefore, selective inhibition of p38 MAPK may also prove to be an effective anti-inflammatory therapeutic strategy in CF.

As oxidative stress and oxidant-driven inflammation is such a prominent component of these diseases, which can not only alter drug targeting but may also induce glucocorticoid insensitivity, it is critical to assess if these inhibitors are able to suppress oxidant-driven inflammatory responses where glucocorticoids fail.[69,127] Encouragingly, inhibition of p38 MAPK α also reduces the inflammatory response in cigarette smoke-mediated glucocorticoid-insensitive models *in vivo*.[128] In addition, inhibition of p38 MAPK may also improve glucocorticoid function in PBMCs from COPD patients.[129]

Consequently, direct inhibition of p38 MAPK has been proposed as a potential novel anti-inflammatory strategy and a number of selective p38 MAPK α inhibitors are currently under development for respiratory disease.[122]

5.1.2 PI3Kδ/γ

The lipid kinases PI3Kδ and PI3Kγ have a relatively restricted expression to mast cells and leukocytes, and are central in mediating both the innate and adaptive inflammatory responses.[130] Selective inhibition of these PI3K isoforms has proven particularly successful in models of allergic inflammation by reducing airway hyperresponsiveness, eosinophilia and mast cell degranulation, as well as B- and T-cell function.[130-135] Consequently, a number of small-molecule inhibitors of these isoforms are currently under development for asthma.[136]

In contrast to allergy, the roles of these isoforms in diseases that are driven by innate (T_h1) immunity such as COPD and CF, or that have a T_h1 component such as severe asthma, have not

been directly studied. However, the impact of these isoforms on the key cells, including neutrophils and macrophages, involved in these diseases has been investigated. Neutrophil migration, directional chemotaxis and respiratory burst are dependent on PI3Kγ and δ signalling.[134,137,138] Monocyte recruitment into the lungs is also dependant on PI3Kδ signalling.[139] In addition, T_h1 and Tc1 cells are now proposed to be important in the orchestration of chronic inflammatory responses. PI3K signalling also appears to be important in T-cell trafficking and the retention of specific T cell types, such as Tc1 cells.[140]

Therefore, the role of PI3Kγ and δ in the regulation of the innate immune response and the ability to selectively target these isoforms presents an attractive and novel anti-inflammatory therapeutic strategy for the treatment of severe asthma, COPD and CF.

5.2 Therapeutic Targets for the Restoration of Glucocorticoid Function

In addition to novel anti-inflammatory targets, another strategy being pursued is the pharmacological restoration of glucocorticoid function, which would have the benefit of re-establishing an effective anti-inflammatory therapy that was well profiled in terms of application, drug interactions and adverse effects. Restoration of glucocorticoid function has been achieved both in *in vitro* and *in vivo* glucocorticoid-insensitive models and in primary cells from patients with disease through selective inhibition of the PI3Kδ/Akt pathway.[81,107] These targets along with additional strategies are discussed in the following sections.

5.2.1 Antioxidants

As oxidant stress is proposed to play a central role in not only the development and progression of COPD, severe asthma and CF but also in the mechanisms of relative glucocorticoid insensitivity, it would be obvious to directly target this oxidant stress with antioxidants. A number of studies have suggested that use of antioxidant supplements such as N-acetyl-cysteine (NAC) or an increase in dietary antioxidants may be beneficial in COPD.[141,142] This is an attractive therapeutic strategy; however, one of the major hurdles is that antioxidants have a poor bioavailability and attempts to deliver an effective concentration to the lungs has proven difficult, with little clinical benefit seen in COPD to date.[143,144] If bioavailability and delivery hurdles are overcome and antioxidant drugs such as NAC are able to be delivered into the lung at an effective concentration, then it is likely that they would have a beneficial impact on the function of inhaled glucocorticoids.

5.2.2 Long-Acting β_2 Agonists

Long-acting β_2 agonists (LABAs) are bronchodilator drugs used in both asthma and COPD. Although their use as a monotherapy is controversial, their use as a combination therapy with inhaled glucocorticoids has been shown to have several beneficial effects, including improved glucocorticoid function.[45,145] When inhaled glucocorticoid therapy alone fails to control asthma, addition of a LABA as a combination therapy improves symptom score, improves lung function, reduces the use of rapid-acting β_2 agonists and reduces the number of exacerbations.[45] Several large-scale studies have demonstrated that use of LABAs in combination with inhaled glucocorticoids in patients with moderate-to-severe COPD improved lung function (although it does not influence its long-term deterioration), exacerbations, breathlessness and health status.[145]

The exact molecular mechanisms of this improved glucocorticoid function remain unclear, but it is thought that LABAs may improve the penetration of inhaled glucocorticoids into lung cells. Although the evidence is clear that a combination therapy of LABA-inhaled glucocorticoid improves symptoms and lung function in both asthma and COPD compared with inhaled glucocorticoids alone,[146] there is little evidence to suggest that their use results in a substantial improvement in glucocorticoid immunosuppression and ultimate disease progression. Therefore, the additional use of an effective anti-inflammatory is still required in these diseases.

5.2.3 Theophylline

Several studies have shown that the first-generation bronchodilator theophylline used at low concentrations restored glucocorticoid function in an HDAC-2-dependent manner.[92,127,147,148] Enhancement of glucocorticoid function by theophylline has been seen clinically for a number of years where it has been used in patients with moderate to severe asthma.[147,149] However, although theophylline may act through a number of pathways including that of PI3Kδ/Akt (discussed in section 5.2.4), the exact target(s) by which theophylline acts at these low concentrations has not yet been established. Interestingly, the binding profile of theophylline is altered under conditions of oxidative stress, which may explain why it has no apparent impact on HDAC-2 activity in non-oxidant stressed cells.[127] Importantly, this finding may also have wider implications in terms of drug binding and targeting in diseases with a prominent component of oxidative stress and should be taken into consideration in the development of therapies for these diseases.

5.2.4 PI3Kδ

In models of oxidant-mediated glucocorticoid insensitivity, inhibition of the PI3K/Akt pathway also restores glucocorticoid function in an HDAC-2-dependent manner.[81] Our studies using selective small molecule inhibitors and transgenic mice in oxidant-mediated glucocorticoid insensitivity have revealed that it is the PI3Kδ isoform that is responsible for this action.[81] Furthermore, selective inhibition of PI3Kδ restores glucocorticoid function in primary cells from COPD patients as compared with age-matched smoking controls.[107] The precise mechanisms by which inhibition of PI3Kδ signalling restores glucocorticoid function is not clear but appears to involve the protection of HDAC-2 activity by reducing oxidant-mediated hyperphosphorylation and nitration.[81] In addition, abolition of PI3Kδ and γ signalling also protects the expression of other key components of the HDAC-2-containing co-repressor complexes (including Mi2 expression), which are recruited by GRα to mediate gene repression. However, PI3Kδ signalling is an early event occurring at the inner phospholipid layer of the cell surface membrane and is therefore not likely to directly interact with HDAC-2 or other co-repressors. PI3Kδ signalling is activated by oxidative stress and is the major PI3K isoform responsible for activation of Akt.[107,150] Akt may thereafter act directly or on signalling kinases further downstream, although extensive investigations are still needed to elucidate the signalling pathways that link oxidant activation of PI3Kδ/Akt and their protective effects on HDAC-2/co-repressor expression and activity.

Therefore, as small-molecule inhibitors of PI3Kδ and dual inhibitors of PI3Kδ/γ are currently being considered for allergic disease, these findings represent a significant development in a possible therapeutic strategy for the restoration of glucocorticoid function in COPD, which may also be applicable to severe asthma and CF.

6. Conclusions

Relative glucocorticoid-insensitive inflammation is an unmet medical need in a number of diseases, including severe asthma, COPD and CF. The clinical significance of this relative glucocorticoid insensitivity in these diseases, which include disease management problems and cost burden, has led to increasing investigation into new effective anti-inflammatory strategies in what has been an understudied area.

Development of inhibitors against novel targets with broad-ranging anti-inflammatory functions such as p38 MAPK α and PI3Kδ/γ, which may also act to restore the function of glucocorticoids, may provide an effective therapeutic strategy against the enhanced glucocorticoid-insensitive inflammation seen in these diseases. However, despite the identification of the importance of oxidant-mediated alterations in kinase signalling and co-repressor expression/activity, the precise mechanism(s) of the relative glucocorticoid insensitivity in these diseases still remain unknown. Due to the complexity of the inflammatory responses in these diseases, which include components of both the innate and adaptive immune systems, oxidant stress and

protease imbalance, it is likely that the mechanism(s) by which glucocorticoid function is impaired will be complex and multifaceted. However, given the similarities in aspects of the inflammation present in the lungs of patients with severe asthma, COPD and CF, there may be common mechanisms (such as those mediated by oxidant stress) that may provide a viable therapeutic strategy applicable across these diseases. It is therefore important that all of the potential mechanisms of relative glucocorticoid insensitivity in these diseases are elucidated to provide a clearer picture from which effective alternative anti-inflammatory and/or glucocorticoid-restorative therapies may be developed.

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